

Association of *MC1R* Variants and Risk of Melanoma in Melanoma-Prone Families with *CDKN2A* Mutations

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Abstract

Major risk factors for melanoma include many nevi, especially dysplastic nevi, fair pigmentation, freckling, poor tanning ability, and germ line mutations in the *CDKN2A*, *CDK4*, or *MC1R* genes. We evaluated the relationship between *MC1R* and melanoma risk in *CDKN2A* melanoma-prone families with extensive clinical and epidemiologic data. We studied 395 subjects from 16 American *CDKN2A* families. Major melanoma risk factors were assessed by clinical examination or questionnaire; *MC1R* was sequenced. Odds ratios were estimated by unconditional and conditional logistic regression models. We examined the distribution of *MC1R* variants and median ages at melanoma diagnosis in multiple primary melanoma (MPM) and single primary melanoma (SPM) patients. Presence of multiple *MC1R* variants was significantly associated with melanoma, even after adjustment for

major melanoma risk factors. All 40 MPM patients had at least one *MC1R* variant; 65% of MPM patients versus only 17% of SPM patients had at least two *MC1R* variants ($P < 0.0001$). For all 69 melanoma patients combined, as well as the 40 MPM patients, there was a statistically significant decrease in median age at diagnosis as numbers of *MC1R* variants increased ($P = 0.010$ and $P = 0.008$, respectively). In contrast, no significant reduction in age at melanoma diagnosis was observed for SPM patients ($P = 0.91$). The current study suggests that the presence of multiple *MC1R* variants is associated with the development of multiple melanoma tumors in patients with *CDKN2A* mutations. Additional studies are needed to confirm these findings and to explore the mechanisms that may contribute to this relationship. (Cancer Epidemiol Biomarkers Prev 2005;14(9):2208–12)

Introduction

Cutaneous malignant melanoma (CMM) is a potentially fatal form of skin cancer whose etiology is heterogeneous and complex. Major host and environmental risk factors for melanoma include many melanocytic nevi, especially dysplastic nevi, fair pigmentation (skin, eye, and hair), freckling, poor tanning ability, and a tendency to burn after sun exposure (1, 2). Genetic risk factors include germ line mutations in the *CDKN2A*, *CDK4*, or *MC1R* genes. *CDKN2A* and *CDK4* have been designated “high-risk” melanoma susceptibility genes. The *CDKN2A* gene is the major known melanoma susceptibility gene. Germ line mutations have been detected in ~20% of melanoma-prone families with three or more melanoma patients. In contrast, few families with germ line mutations in *CDK4* have been identified. *MC1R* has also been shown to influence melanoma risk, but it is described as a “low risk” melanoma susceptibility gene (3, 4).

MC1R is involved in pigmentation primarily through its binding with α -melanocyte-stimulating hormone (5). *MC1R* is very polymorphic, with >65 nonsynonymous alleles identified to date (6, 7). Three variants (*R151C*, *R160W*, and *D294H*) designated red hair color or *RHC* variants have been repeatedly shown to be associated with red hair color, poor tanning ability, pale/fair skin color, and extensive freckling (8–10). Most other variants (designated *non-RHC* or *NRHC*) have a weaker or no association with red hair (10, 11). Several studies

conducted in generally fair-skinned populations of Northern European origin have shown that risk of melanoma is higher among *MC1R* variant carriers than among noncarriers, with the strongest effects observed for carriers of multiple variants (9, 12, 13).

MC1R has also been shown to be a risk factor for melanoma in families segregating *CDKN2A* mutations. A study of 15 Australian *CDKN2A* mutation-carrying families with nine different mutations (14) and a study of 101 *p16-Leiden* mutation carriers from six Dutch families (15) both showed that the presence of *MC1R* variants increased the frequency/penetrance of melanoma among *CDKN2A* mutation carriers. The *MC1R*-melanoma association was primarily related to the *R151C* variant in the Dutch families and to the three *RHC* variants in the Australian families. There was also an inconsistent reduction in age at melanoma diagnosis associated with the presence of at least one *MC1R* variant; this age reduction was observed in the Australian study sample but not in the Dutch families. Further studies are needed to confirm and refine the findings from the Australian and Dutch *CDKN2A* families. The objective of the current study was to evaluate the relationship between *MC1R* and melanoma risk in 16 *CDKN2A* melanoma-prone American families with extensive clinical and epidemiologic risk factor data.

Materials and Methods

Participants and Design. Families were recruited for this non-population-based family study if there was a history of invasive melanoma in at least two first-degree relatives. The families were referred by health-care professionals or through self-referrals. Written informed consent was obtained prior to participation under an Institutional Review Board–approved protocol. All family members willing to participate in the study were clinically evaluated. Variables recorded during the clinical examination included the type and total number of

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nevi, extent of freckling, skin complexion, evidence for solar injury, and hair and eye color. In addition, a self-administered questionnaire obtained information on sun-related variables such as the skin's reaction to acute and chronic sun exposure (i.e., tanning ability). The subjects for this study were drawn from 16 families in which a *CDKN2A* mutation had been previously identified (16). The families had the following 12 mutations: *1_8dup8*, *L16R*, *M53I*, *R58X*, *N71S*, *R87P*, *S56fs* (*c.167_197del31*), *c.240_253del14*, *P75fs* (*c.225_243del19*), *G101W* (*n* = 3), *V126D* (*n* = 3), and *c.IVS2+1 G>T*. The families have been followed prospectively from 4 to 26 years starting in the 1970s. All melanoma diagnoses were confirmed by review of histologic materials, pathology reports, medical records, or death certificates. Total numbers of primary melanomas were recorded for each melanoma patient.

Sequencing of *MC1R*. *MC1R* genotyping was conducted at the National Cancer Institute, Frederick, MD employing PCR amplification of the 951 bp coding region of *MC1R*, either in its entirety or in smaller overlapping segments, followed by complete direct sequencing of the amplicon(s). The coding region of *MC1R* was amplified from genomic DNA extracted from patient blood samples using two sets of M13-tagged PCR primers *MC1R_1F*: 5'-GTA AAA CGA CGG CCA GTG AAG ACT TCT GGG CTC CCT C-3'; *MC1R_IIIR*: 5'-GGA AAC AGC TAT GAC CAT GGC GTG CTG AAG ACG ACA CT-3'; and *MC1R_IVF*: 5'-GTA AAA CGA CGG CCA GTG TGC TGT ACG TCC ACA TGC T-3'; *MC1R_IVR*: 5'-GGA AAC AGC TAT GAC CAT GCT CTG CCC AGC ACA CTT AAA-3'. The underlined region of the primer is specific to the target DNA. The reaction mix for PCR amplification included 1× PCR buffer (Invitrogen high-fidelity PCR buffer), 1.5 mmol/L $MgSO_4$, 175 nmol/L each pair of primers, 50 nmol/L each of the four deoxynucleotide triphosphates, and 1 unit of HiFi Platinum Taq polymerase (Invitrogen, Carlsbad, CA). All PCR products were processed prior to sequencing. All products from two regions of PCR were sequenced with ABI prism BigDye terminator cycle sequencing kit 1.0 (Applied Biosystems, Inc.) on ABI3700 sequence analyzer using sequence primers *1F*: 5'-GCT CCC TCA ACT CCA CC-3'; *IR*: 5'-GAA GAC GAC ACT GGC CAC-3' and *M13F*: 5'-GTA AAA CGA CGG CCA GT-3'; *M13R*: 5'-GGA AAC AGC TAT GAC CAT G-3', respectively. All sequences were analyzed and variants were detected using Mutation Surveyor (SoftGenetics Inc., PA) and sequence analysis software package developed at the Laboratory of Molecular Technology, National Cancer Institute.

Statistical Analyses. Initially, we evaluated each *MC1R* variant individually comparing 1+ variant to the consensus *MC1R* sequence (i.e., wild-type *MC1R*). Because many *MC1R* variants were too rare to examine their individual associations with melanoma risk after adjustment for major melanoma risk factors (i.e., *CDKN2A* status, nevus/pigmentation factors—see below), we also used the following *MC1R* variables in the analyses: carriers of any *MC1R* variant compared with wild-type *MC1R*; carriers of multiple (1, 2+) variants compared with the consensus sequence; carriers of 1 NRHC variant, 2+ NRHC variants, 1 RHC variant, 2+ RHC variants, or carriers of both RHC and NRHC variants compared with wild-type *MC1R*.

For purposes of this study, the measure of association between melanoma risk and the clinical, genetic, and environmental variables was the odds ratio (OR). Point estimates and 95% confidence intervals (CI) of adjusted ORs were calculated using logistic regression analysis as implemented in the EPICURE package (17).

We assessed the association of pigmentation and nevus characteristics with all nonsynonymous *MC1R* variants combined using χ^2 and Fisher exact tests in the unaffected relative and spouse controls separately (Stata 8.2; ref. 18). Dysplastic nevi, hair color, eye color, skin complexion, freckling, solar

injury, and tanning ability were all associated with *MC1R*. We also evaluated the ORs between these same factors and melanoma risk. Because of the relatively small number of cases, we created summary factors that combined the covariates showing the strongest associations with both *MC1R* variants and melanoma risk. A three-category nevus factor was created by combining dysplastic nevi (absent, indeterminate, present) and total numbers of nevi. Similarly, a three-category pigmentation factor was developed by combining skin complexion (medium/dark, pale/fair) and extent of freckling (none/few, moderate, many).

We conducted two logistic regression analyses (17). The first analysis conditioned on family membership using the entire data set (72 melanoma cases, 245 unaffected relative, and 78 spouse controls). We also conducted an unconditional logistic regression analysis on the subset of confirmed *CDKN2A* mutation carriers (69 cases and 72 unaffected relative controls). All analyses were adjusted for age as a continuous variable. Sex had no effect on risk of melanoma and therefore was excluded from all analyses (data not shown). For the conditional logistic regression analysis, three models were examined: univariate (with age adjustment); adjustment for *CDKN2A* status and age; and adjustment for age, *CDKN2A*, and the pigmentation/nevus factors. For the unconditional analysis of *CDKN2A* mutation carriers, two models were evaluated: univariate (adjusted for age) and adjustment for age and pigmentation/nevus factors.

We examined the distribution of *MC1R* variants in multiple primary melanoma (MPM) compared with single primary melanoma (SPM) patients using Fisher exact test as implemented in StatXact 4 (19). We also estimated the median ages at diagnosis of initial melanomas in all melanoma patients and in MPM and SPM patients separately. The nonparametric Jonckheere-Terpstra test was used to investigate the hypothesis of no differences among the ages at diagnosis of melanoma according to numbers or numbers/types of *MC1R* variants against the alternative that the ages at diagnosis decreased as the numbers or numbers/types of *MC1R* variants increased.

Results

Ten nonsynonymous and five synonymous (i.e., silent) *MC1R* variants were detected in the 395 subjects sequenced for *MC1R*. Table 1 shows the number of cases, unaffected relative controls, and spouse controls with each of the nonsilent variants observed. The five silent variants found (*R34R*, *A166A*, *A240A*, *I264I*, and *T314T*) were excluded from all analyses. The most frequent variants observed were *V60L*, *R160W*, and *R151C*. As has been previously observed in other studies, there was a strong association between the RHC variants *R151C*, *R160W*, and *D294H* and red hair color. Seventy-seven percent of the subjects (20 of 26) with red hair had at least two RHC variants. Also, only 29% of the subjects (8 of 28) without red hair had two RHC variants. These eight subjects had primarily brown or light brown hair. In contrast, few subjects with *V60L*, *V92M*, *I155T*, or *R163Q* had red hair color.

Table 1 presents ORs between melanoma risk and individual *MC1R* variants. After conditioning on family membership and adjusting for age, there were significant associations between melanoma and the presence of *R151C* or *R160W*. Unconditional analyses restricted to *CDKN2A* mutation carriers showed significant associations between melanoma risk and all *MC1R* variants evaluated except for *86insA* and *I155T*, but with wide confidence intervals.

Table 2 shows the associations between melanoma risk and selected *MC1R* covariates. Table 2A presents the ORs and 95% CIs for all three conditional analysis models evaluated. For the three analyses, the presence of at least two *MC1R* variants was significantly associated with melanoma [OR, 5.6 (2.1-14.7);

Table 1. Number of cases, unaffected relative and spouse controls with MC1R variants, ORs and 95% CIs for individual MC1R variants and risk of melanoma

MC1R variants	Cases (n = 72)	All unaffected relative controls (n = 245)	Unaffected relative control mutation carriers (n = 72)	Spouse controls (n = 78)	No. of informative families	All-subjects analysis (conditioning on family), *OR (95% CI)	Subset analysis of CDKN2A mutation carriers,*OR (95% CI)
None (wild-type)	6 [†]	62	23	23		—	—
86insA	2	7	3	2	4	1.7 (0.2-18.2)	3.4 (0.4-28.9)
V60L	23 [†]	75	15	17	10	2.6 (0.9-7.6)	8.2 (2.2-30.3)
S83P	1	1	1	1		—	—
D84E	0	7	3	5		—	—
V92M	11	33	9	8	10	1.6 (0.4-6.9)	12.3 (2.3-66.5)
R151C [‡]	15 [†]	42	10	10	11	6.0 (1.4-26.3)	8.6 (2.1-34.5)
I155T	5	7	5	2	3	—	4.1 (0.8-21.0)
R160W [‡]	19	35	4	14	9	3.4 (1.2-9.8)	26 (5-130)
R163Q	5	13	3	7	6	4.3 (0.6-28.6)	10.2 (1.6-66.3)
D294H [‡]	7	13	5	3	5	1.3 (0.1-13.9)	15.6 (2.1-114.4)

*All ORs adjusted for age.
†One case with this MC1R variant was not a CDKN2A mutation carrier.
‡RHC variants.

OR, 20 (5-80); and OR, 6.1 (1.2-29.7), respectively]. Any MC1R variant, the number of variants, and types of variants also showed significant but imprecise associations with melanoma when we adjusted for age only or age and CDKN2A status. R151C and R160W also showed significant associations with CMM after adjustment for both age and CDKN2A status [OR, 11.3 (1.4-93.3) and OR, 9.1 (1.6-52.4), respectively]. Table 2B

shows the number of cases and unaffected relative controls who were CDKN2A mutation carriers and results from the unconditional subset analysis of CDKN2A mutation carriers. There were significant associations between melanoma risk and all three summary MC1R variables examined after adjustment for age only. In addition, after adjustment for age and the pigmentation/nevus factors, there were significant

Table 2. Number of cases, unaffected relative, and spouse controls, ORs and 95% CIs for selected MC1R variants and risk of melanoma

MC1R variables	Cases	Unaffected relative controls	Spouse controls*	Adjustment for age only, OR (95% CI)	Adjustment for age and CDKN2A, [†] OR (95% CI)	Adjustment for age, CDKN2A, pigmentation/ nevus factors, OR (95% CI)
(A) All-subjects analysis (conditioning on family)						
Any variant						
No	6	62	22	—	—	—
Yes	66	183	56	3.6 (1.4-8.8)	6.9 (2.0-23.4)	1.9 (0.5-7.4)
No. of variants						
0	6	62	22	—	—	—
1	35	116	39	2.8 (1.1-7.1)	4.6 (1.3-15.9)	1.1 (0.3-4.7)
≥2	31	67	17	5.6 (2.1-14.7)	20 (5-80)	6.1 (1.2-29.7)
Types of variants						
None	6	62	22	—	—	—
1 NRHC	19	79	24	2.2 (0.8-6.0)	2.9 (0.7-11.2)	1.0 (0.2-4.4)
2+ NRHC	9	18	8	4.9 (1.5-16.1)	12.3 (2.0-76.2)	4.3 (0.5-35.3)
1 RHC	16	37	15	3.8 (1.3-10.6)	8.9 (2.2-36.4)	1.5 (0.3-7.7)
1 RHC and 1 NRHC	15	33	5	6.1 (2.1-17.9)	22 (4-105)	6.0 (1.0-37.0)
2+ RHC	7	16	4	5.5 (1.6-18.8)	55 (8-384)	13 (2-119)
(B) Subset analysis of CDKN2A mutation carriers (unconditional analysis)						
				Adjustment for age only OR (95% CI)		Adjustment for age, pigmentation/nevus factors OR (95% CI)
Any variant						
No	5	23		—		—
Yes	64	49		9.3 (2.9-30.1)		3.1 (0.8-11.5)
No. of variants						
0	5	23		—		—
1	33	39		5.9 (1.7-19.8)		1.7 (0.4-7.1)
≥2	31	10		24 (6-92)		7.3 (1.6-33.2)
Types of variants						
None	5	23		—		—
1 NRHC	18	26		4.5 (1.2-16.0)		1.5 (0.4-6.6)
2+ NRHC	9	4		13.7 (2.5-74.2)		7.1 (1.0-49.4)
1 RHC	15	13		9.9 (2.4-40.9)		2.3 (0.5-11.8)
1 RHC and 1 NRHC	15	6		23 (5-106)		5.7 (1.0-32.2)
2+ RHC	7	0		—		—

*No spouse controls have CDKN2A mutations and were excluded from analyses of CDKN2A mutation carriers.
†Model not applicable for subset analysis of CDKN2A mutation carriers.

associations between melanoma and multiple *MC1R* variants [OR, 7.3 (1.6-33.2)] as well as suggestive associations with the presence of at least two *NRHC* variants [OR, 7.1 (1.0-49.4)] or the presence of both *RHC* and *NRHC* variants [OR, 5.7 (1.0-32.2)]. These analyses were, however, based on relatively small numbers that resulted in wide confidence intervals. It was not possible to fully evaluate the number of *RHC* variants. Specifically, seven cases and no controls had two *RHC* variants.

Table 3 shows the number of *MC1R* variants in MPM and SPM patients with *CDKN2A* mutations. There were three SPM patients who were not *CDKN2A* mutation carriers; these patients were excluded from the MPM-SPM evaluations. There were significant differences in the distribution of *MC1R* variants between MPM and SPM patients. All 40 MPM patients had at least one *MC1R* variant; 65% of MPM patients versus only 17% of SPM patients had at least two *MC1R* variants ($P < 0.0001$). Multiple *NRHC* variants and presence of both *RHC* and *NRHC* variants were more frequent in MPM versus SPM patients. Variation in other major melanoma risk factors including freckling, hair color, eye color, tanning ability, total nevi, or dysplastic nevi did not explain the differences in *MC1R* covariates in MPM versus SPM patients (data not shown).

Table 4 shows the median age at first melanoma diagnosis for SPM, MPM, and all *CDKN2A* mutation-carrying CMM patients according to the number of *MC1R* variants or numbers/types of *MC1R* variants. For all 69 patients combined, there was a statistically significant decrease in median age at diagnosis as the number of *MC1R* variants increased ($P = 0.010$) even considering *RHC* and *NRHC* variants separately ($P = 0.003$). This reduction in median age at CMM diagnosis in all *CDKN2A* mutation-carrying melanoma patients was primarily because of a significant decrease in age at diagnosis in MPM patients. No significant reduction in age at melanoma diagnosis was observed for SPM patients (Table 4).

Discussion

We examined the association between *MC1R* variants and melanoma risk in 16 melanoma-prone American families with *CDKN2A* mutations. Similar to what has been observed in other *CDKN2A* mutation-carrying melanoma-prone families (14, 15, 20), we observed a significant association between increased numbers of *MC1R* variants and melanoma risk even after adjustment for major melanoma risk factors. In addition, comparison of MPM and SPM patients revealed striking

Table 3. Distribution of *MC1R* variants in *CDKN2A* mutation-carrying MPM and SPM patients

	No. of CMM <i>CDKN2A</i> mutation carriers		Fisher's exact <i>P</i> value
	MPM	SPM	
Any <i>MC1R</i> variant			
No	0	5	0.011
Yes	40	24	
No. of variants			
0	0	5	<0.0001
1	14	19	
≥2	26	5	
Types of variants			
None	0	5	0.0015
1 <i>NRHC</i>	7	11	
2+ <i>NRHC</i>	8	1	
1 <i>RHC</i>	7	8	
1+ <i>RHC</i> and 1+ <i>NRHC</i>	13	2	
2+ <i>RHC</i>	5	2	

Table 4. Median ages at melanoma diagnosis in MPM, SPM, and all *CDKN2A* mutation-carrying (*CDKN2A*+) melanoma patients

	Median ages at melanoma diagnosis		
	MPM	SPM	All <i>CDKN2A</i> + CMM patients
No. of variants			
0	—	36	36
1	37.5	31	32
≥2	24	36	27
<i>P</i> value*	0.008	0.91	0.010
Types of variants			
None	—	36	36
1 <i>NRHC</i>	27	34	33
2+ <i>NRHC</i>	31	56	31
1 <i>RHC</i>	38	26	31
1 <i>RHC</i> and 1 <i>NRHC</i>	23	28	27
2+ <i>RHC</i>	19	39	22
<i>P</i> value*	0.001	0.32	0.003

*Jonckheere-Terpstra test.

differences in the distributions of *MC1R* variants in these two groups of patients. There were also significant differences in median ages at melanoma diagnosis according to numbers and/or types of *MC1R* variants in all *CDKN2A* mutation-carrying melanoma patients and MPM patients.

To the best of our knowledge, this is the first study of *MC1R* variants in *CDKN2A* mutation carriers that examined the relationship between MPM and SPM patients from the same study sample. The MPM findings observed here are further supported by a small Italian study of 14 MPM patients without a positive family history for melanoma; Peris et al. (21) detected *MC1R* variants in 11 of 12 patients with nonfamilial MPM, a much higher frequency relative to that previously reported in other populations (22). Two of the patients with *MC1R* variants also had *CDKN2A* mutations as well as red hair color. The authors suggested that the results might represent an example of the effects of gene-gene interaction on disease risk (21). The current study with thrice the number of MPM patients plus 29 SPM patients, all with *CDKN2A* mutations, suggests that the presence of multiple *MC1R* variants is associated with the development of multiple melanoma tumors in patients with *CDKN2A* mutations. Although the small sample size precludes full evaluation of this association, the dampening of the complex host risk with sun-related factors (i.e., freckling/multiple nevi/dysplastic nevi) hints at the possible importance of sun exposure. Additional studies are needed to confirm these findings and to explore the mechanisms that may contribute to this relationship.

The Australian and Dutch studies of *MC1R* variants in melanoma-prone families with *CDKN2A* mutations showed inconsistent differences in age at melanoma diagnosis. In the Australian study, mean age at melanoma diagnosis decreased significantly from 58.1 to 37.8 years with the presence of one or more *MC1R* variants (14). In contrast, the Dutch study showed no such reduction in age at diagnosis; in fact, the mean age at melanoma diagnosis was 40 years in melanoma patients with no *MC1R* variants and 42 to 45 years in patients with two or more *MC1R* variants (15). The current study revealed a significant decrease in median age at melanoma diagnosis as the overall number of *MC1R* variants increased and when looking at the number of *RHC* and *NRHC* variants separately. However, this association resulted from melanoma patients with >1 melanoma tumor (i.e., MPM patients). That is, among the 29 patients with only one melanoma tumor, there was no significant association between *MC1R* variants and age at melanoma diagnosis. It is possible that differences in the number of MPM versus SPM patients between the Dutch and

Australian studies may have contributed to the inconsistent results observed in these two studies. Alternatively (or in addition), differences in the types or distribution of *CDKN2A* mutations across the two studies—nine *CDKN2A* mutations in the Australian study versus one founder mutation (*p16-Leiden*) in the Dutch study—might have influenced the ages at melanoma diagnosis and/or the development of MPM tumors. Finally, distribution of major melanoma risk factors including relative amounts of sun exposure and the skin's reaction to sun exposure may have differed between the two studies. Further studies are needed to evaluate the age association between *MC1R* and numbers of melanoma tumors (and sun exposure).

The current study was limited by the small number of confirmed mutation carriers. The small size precluded adjustment for family membership in the *CDKN2A* mutation carrier subset analysis. In addition, it was not possible to examine individual *CDKN2A* mutations or *CDKN2A* mutations classified according to their type, location, or effect on the p14ARF protein. Also, it was difficult to evaluate *MC1R* variants separately. In addition, even though significant associations between melanoma risk and multiple *MC1R* variants were observed after adjustment for major melanoma risk factors, the odds ratio estimates were imprecise with wide confidence intervals. Finally, although all family members were invited to participate in the study, differential inclusion of mutation carriers, deceased melanoma cases or relatives with certain exposures could influence the results. It is difficult, however, to predict whether the odds ratios would be decreased or increased by this potential participation bias. In conclusion, this study of 16 melanoma-prone American families with *CDKN2A* mutations adds to the growing literature of studies demonstrating a relationship between multiple *MC1R* variants and melanoma risk. The study also provides new directions for research to further explore the differences in the distribution of *MC1R* variants and ages at melanoma diagnosis observed in MPM versus SPM patients. Studies with much larger sample sizes and extensive epidemiologic, clinical, and genetic risk factor data will be required to investigate these relationships further.

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